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SULFIDE OXIDATION OCCURS IN THE ANIMAL TISSUE OF THE GUTLESS CLAM, *SOLEMYA REIDI*

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Abstract

A gutless clam, *Solemya reidi,* from sulfide-rich habitats has gills containing symbiotic, chemoautolithotrophic bacteria that are presumed to oxidize sulfide to provide a major energy source for the symbiosis. Sulfide oxidation was studied for *S. reidi;* activity of gill and foot extracts displayed Michaelis-Menten kinetics and was presumably due to sulfide oxidase enzymes. The activity of *S. reidi* gill extracts was protease sensitive and heat sensitive and was 10 to 20 times higher than in tissues from bivalves not living in sulfide-rich environments. The site of sulfide oxidation was studied by cytochemistry, ^{35}S-sulfide autoradiography, cell fractionation methods, and by X-ray microanalysis. In gills of *S. reidi,* the sulfide-oxidizing activity was detected not in the symbiotic bacteria, but within organelles of the gill cells we have named sulfide oxidizing bodies. In foot tissue of *S. reidi,* sulfide oxidation activity was distributed diffusely throughout the superficial cell layers of the foot. The discussion considers the roles of sulfide oxidation in protection of aerobic respiration from sulfide inhibition and in exploitation of the energy contained in sulfide.

Introduction

A variety of marine invertebrates (pogonophoran tube worms, bivalve molluscs, and oligochaetes) from sulfide-rich habitats harbor sulfur bacteria as symbionts (Cavanaugh *et al.,* 1981; Felbeck *et al.,* 1981; Cavanaugh, 1983; Felbeck *et al.,* 1983; Fisher and Hand, 1984). These bacteria have enzymes of the Calvin-Benson cycle (Felbeck, 1981; Felbeck *et al.,* 1981; Felbeck and Somero, 1982; Felbeck, 1983; Felbeck *et al.,* 1983; Fisher and Hand, 1984), and the energy (reducing power and ATP) needed for net CO_2 fixation, as well as for reducing nitrate and nitrite, is thought to come from the exergonic oxidation of sulfide to, *e.g.,* sulfate (*cf.* Jannasch, 1984). Enzymes involved in ATP- and NAD(P)H-generating sulfide oxidation pathways have been found in these symbioses (*cf.* Felbeck *et al.,* 1981).

In addition to possessing the enzymic pathways for a sulfide-based chemoautolithotrophic type of metabolism, these symbioses are likely to depend on a second important type of adaptation to sulfide, mechanisms which prevent the poisoning of aerobic respiration by sulfide. Micromolar amounts of hydrogen sulfide are extremely toxic to most organisms because it inhibits the cytochrome c oxidase system (National Research Council, 1979). Yet in the habitats where these symbioses have been found, sulfide concentrations range between about 100 μM and 20 mM (Edmond *et al.,* 1982; Felbeck, 1983). Because levels of cytochrome c oxidase activity in these symbioses and in related species from sulfide-free habitats are similar, it appears that these symbioses are able to carry out aerobic respiration (Hand and Somero, 1983; Powell and Somero, 1983).

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Our initial study of the mechanisms that prevent poisoning by sulfide of aerobic respiration focused on the hydrothermal vent vestimentiferan tube worm, *Riftia pachyptila* (phylum Pogonophora) (Powell and Somero, 1983). We showed that the respiration of obturacular plume tissue in the presence of high concentrations of sulfide was not due to an inability of sulfide to penetrate this tissue or to a sulfide-insensitive form of cytochrome c oxidase. Instead, we showed that a component of the worm's blood, probably the sulfide binding protein (Arp and Childress, 1983), was responsible for protecting cytochrome c oxidase activity. The sulfide binding protein does not function as a sulfide oxidase. Instead, it binds sulfide very tightly, and acts as a sulfide transport mechanism (Arp and Childress, 1983) and as a means for preventing sulfide concentrations in the animals' cells from reaching levels inhibitory to aerobic respiration (Powell and Somero, 1983).

Despite the importance of the sulfide binding protein for protecting aerobic respiration from sulfide in *Riftia pachyptila,* we reasoned that other means for keeping free sulfide from contact with cytochrome c oxidase are also apt to be critical for animals of sulfide-rich habitats. For example, cells of poorly perfused tissues may not have an adequate amount of blood-borne sulfide binding protein to complex the sulfide entering the tissue. Also, not all animals inhabiting sulfide-rich habitats possess sulfide binding proteins (A. J. Arp and J. J. Childress, pers. comm.). In these cases, it may be essential for the animal's cells to convert sulfide to a non-toxic sulfur compound. In fact, even in tissues with symbionts it is unclear how sulfide from the blood or seawater is provided to the bacteria without the concomitant inhibition of mitochondrial respiration in the symbiont-containing cells. Do enzyme systems in the animal cytoplasm first detoxify sulfide and, then, transport to the bacteria a partially oxidized form of sulfur?

Because of the uncertainties about how and where sulfide is metabolized in these sulfide biome symbioses, we studied sulfide metabolism in the clam, *Solemya reidi.* This animal is a gutless, protobranch bivalve which inhabits sulfide-rich zones like sewage outfall areas (Felbeck, 1983) and pulpmill effluent sites (Reid, 1980). It contains large numbers of bacterial symbionts in its gill, where enzymes of sulfur metabolism and the Calvin-Benson cycle are found (Felbeck, 1983); it has high levels of sulfide-sensitive cytochrome c oxidase activity in gill and foot tissues (Hand and Somero, 1983, unpub. obs. of authors); and it lacks a circulating sulfide-binding protein (A. J. Arp and J. J. Childress, pers. comm.).

Our results suggest that in *S. reidi* gills the initial steps in sulfide oxidation occur in specific organelles in the gill cytoplasm, sulfide oxidizing bodies, not in the bacteria. The implications of this localization of sulfide oxidizing activity for the exploitation of the energy contained in sulfide are discussed. In foot tissue, which lacks bacteria, sulfide oxidizing activities are restricted to the superficial cell layers of the foot muscle. The sulfide oxidizing activities of gill and foot display diagnostic characteristics of enzymes. In both tissues these sulfide oxidizing activities may play important roles in protecting the aerobic respiration of the clam from poisoning by sulfide.

Materials and Methods

Animal collection

Collection of *S. reidi* was by grab sampling of sewage outfall mud near White's Point, California, at a depth of about 100 m. *Mercenaria mercenaria* were purchased from Pt. Loma Seafoods, San Diego, CA. *Chione sp.* were collected from mud flats in Mission Bay, San Diego, CA. *Mytilus edulis* were collected intertidally near Scripps

Institution of Oceanography, La Jolla, CA. Specimens of the teleost fish, *Sebastolobus altivelis*, were collected by trawling, and frozen immediately on dry ice. *S. reidi* were maintained in mud taken from their site of capture, with 8°C seawater slowly flowing over the mud. The other invertebrate species were maintained in flowing seawater at ambient local seawater temperatures (14–18°C). Most experiments were performed with tissues taken from freshly killed animals maintained in the lab less than one month. Animals frozen immediately after capture were used for a few experiments.

Sulfide solutions

Sulfide solutions (usually 100 m*M*) were made fresh using crystals of Na_2S washed in distilled H_2O to remove oxidized material on the surface, and used within several hrs. The term "sulfide" refers to all forms of sulfide present in solution, H_2S, HS^-, and S^{2-}.

Tissue preparation

Tissue samples (1:10 tissue:buffer in most cases; 1:4 when activity was low) were homogenized on ice in 50 m*M* Tris/HCl pH 8.0 (20°C), containing 0.1% Triton X-100. Some homogenates were used uncentrifuged and when clear samples were required (for the spectrophotometric assays) homogenates were centrifuged 10 min at 10,000 $\times g$.

Measurement of sulfide oxidation

Disappearance of sulfide was monitored using a modification of the procedure of Moriarty and Nicholas (1969) with an Orion sulfide-sensitive solid-state microelectrode and microreference electrode connected to an Orion pH/ion electrode meter. The reaction mixture contained 0.80–0.99 ml 40 m*M* glycine buffer pH 9.0, 0–0.1 ml 100 m*M* Na_2S, and 0–0.1 ml sample. All measurements were made aerobically with stirring. The electrode was standardized with solutions of sulfide in 40 m*M* glycine pH 9.0, and rates of sulfide disappearance in the absence of tissue homogenates were measured at various sulfide concentrations. The addition of tissue homogenates shifted the standardization of the electrode so the following procedure was used for measuring sulfide disappearance in the presence of tissue homogenates. First, tissue homogenates were added to the reaction chamber while the buffer was stirred. Second, sulfide was added to the stirred mixture and the initial reading was used as a point in the standardization of the electrode in the presence of tissue homogenates. Third, the decline in sulfide concentration was recorded. This procedure was followed for a number of sulfide concentrations, thus generating a new standard curve for the electrode in the presence of the tissue homogenate being used. Control rates (no homogenate) of sulfide disappearance were subtracted from rates in the presence of tissue homogenates to yield the tissue-specific rates of sulfide oxidation.

The second method used to measure sulfide oxidation involved reduction of the artificial electron acceptor benzyl viologen. Benzyl viologen is colorless when oxidized and intensely purple when reduced. It is rapidly reoxidized by oxygen, so assays must be performed anaerobically. Sulfide levels below 5 m*M* reduce benzyl viologen very slowly in the absence of added tissue homogenates. Higher levels of sulfide lead to an increase in the rate of the non-enzymic reaction, so rates of sulfide oxidation caused by tissue homogenates are best measured using sulfide levels of 5 m*M* and below. The reaction mixture contained 40 m*M* glycine buffer pH 9.0, 2 m*M* benzyl viologen, 0–5 m*M* Na_2S, 0–200 μl sample in a total volume of 1 ml. Reduction of benzyl

viologen was followed at 578 nm using an extinction coefficient of 8.65 absorbance units $\times$ m$M^{-1} \times$ cm^{-1} (McKellar and Sprott, 1979).

Protease treatment

Protease treatment was done at 37°C for 21 h. The reaction mixture consisted of gill extract in 50 m*M* Tris-HCl pH 8.0, 0.1% Triton X-100, and one of the following proteases: pronase (0.5 mg, 4 casein units), trypsin (2.0 mg, 20,000 BAEE units), or chymotrypsin (2.0 mg, 100 BTEE units) in a total volume of 0.1 ml. Proteases were purchased from Sigma Chemical Co.

Activity staining of acrylamide gels

Gels of 7 and 10% acrylamide with 0.1% Triton X-100 added were stained using a modification of the procedure of Jacobson *et al.* (1982) for staining gels of hydrogenase using H_2 and methyl viologen. The gel was carefully inserted into a Ziploc bag and a solution containing 0.2 *M* glycine pH 9.0, 5 m*M* Na_2S, and 2 m*M* benzyl viologen was added. Bubbles were removed, the bag was sealed, and purple colored bands developed in 5–20 min.

Cytochemistry

Sections 6–9 μm thick were cut from unfixed tissue frozen at −70°C in Tissue-Tek II O.C.T. compound and from tissue fixed in 3% glutaraldehyde in 77% seawater for 2 h at room temperature prior to freezing. Sections were stained by addition of 0.2 *M* glycine pH 9.0, 5 m*M* Na_2S, 2 m*M* benzyl viologen made immediately before use. A drop of staining solution was applied to the section and a cover slip was added immediately. Purple color developed in less than 5 min in positively staining areas.

Sections were frozen by addition of a layer of distilled H_2O to sections and insertion of slides into a −20°C freezer until thoroughly frozen. Slides were then thawed at room temperature until no ice was visible.

Toluene treatment consisted of immersion of the section into toluene for several rinses of several min each, followed by an ethanol rinse.

^{35}S-sulfide autoradiography

Clams were used for autoradiography within 30 min of capture. Incubations and tissue fixations were done at sea on board the research vessel R/V Velero IV. The autoradiography buffer consisted of seawater buffered with 20 m*M* HEPES, pH 7.5. Isolated gills were used for the 1 and 10 min incubations, and intact live clams for the 1-h incubation.

The 1-min experiment consisted of isolated gills placed in one ml of buffered seawater at 10°C containing 60 μCi of ^{35}S-sulfide and 1 m*M* total sulfide. After one min the gills were rapidly rinsed twice in 50 ml 10°C buffered seawater lacking sulfide, blotted to remove excess liquid, placed in a foil boat containing sectioning medium, and frozen in liquid N_2 for later sectioning. The total time elapsed between removal from labeled sulfide and freezing was less than 1 min.

The 10-min experiment followed the same procedure except 10 μCi of sulfide were used and the incubation was for 10 min.

The 1-h experiment consisted of small (less than 1 cm) intact live clams incubated in 1 ml of buffered seawater containing 10 μCi of ^{35}S-sulfide and 1 m*M* total sulfide. The valves remained open for most of the incubation period. After 1 h, the clams

were rinsed briefly twice in 10°C buffered seawater lacking sulfide and the gills were removed rapidly, rinsed again twice, blotted to remove excess liquid and frozen as above. Total time elapsed between removal from labeled sulfide and freezing was less than 5 min.

The tissue was frozen for sectioning rather than fixed and embedded because we believed fixation and embedding would be so slow as to allow the diffusion and metabolism of sulfide to continue after the 1-min incubation was over; also, the fixation and embedding process might cause loss of labeled metabolites from the tissue. Four days later, after returning to port, tissues were cut into 6–9 μm sections in a darkroom under a safelight compatible with autoradiography emulsion. Sections were picked up onto Kodak NTB-2 coated slides, placed into slide boxes with desiccant and allowed to expose at 4°C for 8 days. The emulsion was then developed and the slides were mounted.

Isolation of sulfide oxidizing bodies

Gill tissue was homogenized 1:10 in 20 m*M* HEPES pH 7.5, 0.4 *M* sucrose, 1 m*M* EGTA, and 0.5% BSA using a Polytron mechanical homogenizer at ½ speed for 3 bursts of 5 s each with 15 s between bursts to prevent heating of the sample. The sample tube was immersed in an ice and water bath during the entire operation. The sample was then homogenized on ice using a Dounce glass homogenizer, first with a loose, then a tight pestle. Care was taken to avoid foaming and the formation of a vacuum below the pestle. Homogenization was judged to be complete when few intact cells were observed under a microscope.

The sample was then diluted 1:5 with 20 m*M* HEPES pH 7.5, containing 0.4 *M* sucrose and filtered by gravity through a 49 μm Nytex filter with gentle stirring in an Amicon ultrafiltration apparatus. The resulting filtrate was then centrifuged 10 min at 10,000 $\times$ *g*, and the resulting pellet was rehomogenized gently with a Dounce glass homogenizer in a volume of 60% Percoll in HEPES/sucrose equal to about a 1:10 dilution of the original tissue weight.

The mixture was then centrifuged 120 min at 25,000 $\times$ *g* at 4°C in a fixed angle rotor (Sorvall SS-34) to generate a gradient and separate the sample components simultaneously. 0.45-ml fractions were collected from the bottom of the gradient using a peristaltic pump. A tight dark brown band formed near the bottom of the gradient and a chalky brown band formed near the top. Gradients were not monitored for density.

The sulfide-oxidase activity of the fractions was measured. All fractions with substantial sulfide oxidase activity and at least every fourth fraction were counted for bacteria and sulfide oxidizing bodies using a hemocytometer. The counting was simplified by the large size (3–10 μm) and characteristic rod shape of the bacteria. Round structures with a brown-yellow color and 2–5 μm in diameter were counted as sulfide oxidizing bodies. The few nuclei present were clearly distinguishable from sulfide oxidizing bodies.

Microscopy

Tissues were taken from animals within 30 min of capture, and fixed in freshly made 3% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.35, containing 0.35 *M* sucrose, at room temperature for 90 min, and stored at 4°C. After returning to port (4 days after collection), tissues were rinsed 3 times in phosphate/sucrose buffer lacking glutaraldehyde and postfixed for 1 h in 1% OsO_4 in phosphate/sucrose at 0°C. Tissues

were then rinsed in distilled H_2O, dehydrated through graded ethanol solutions, and embedded in Spurr's resin.

Samples used in X-ray microanalysis were not postfixed in OsO_4 because it caused decreased signal to noise ratio during analysis for sulfur.

Sections for light microscopy were cut to 1 or 2 μm, mounted on slides, and stained. Sections for electron microscopy were cut to a thickness of 80–90 A, and stained with uranyl acetate and lead citrate. Sections for X-ray microanalysis were cut to 0.2–1.0 μm, picked up on carbon coated nylon grids, coated with carbon, and used unstained.

RESULTS

Detection of sulfide-oxidizing activity

The two techniques we used for measuring sulfide oxidation, monitoring sulfide disappearance with a sulfide electrode (Moriarty and Nicholas, 1969), and a more sensitive technique which follows the reduction of the artificial electron acceptor benzyl viologen, yielded similar values for the sulfide oxidizing capacity of the tissues we examined. Table I gives data for gill tissue of *Solemya reidi*, the tissue we found to contain the highest amounts of sulfide-oxidizing activity. The variation between assay methods was less than 25%. Since initial studies comparing the two methods showed similar levels of activity, and the benzyl viologen method was much more sensitive and easier to perform, further experiments were conducted using only the benzyl viologen reduction method.

Rates of sulfide oxidizing activity

Because sulfide oxidation occurs spontaneously (non-enzymatically) in seawater containing oxygen, and because metal ions and proteins have been found to stimulate sulfide oxidation, any solution containing tissue extract is liable to have some capacity for oxidizing sulfide (Baxter and Van Reen, 1958; Sorbo, 1960; Chen and Morris, 1972; Almgren and Hagstrom, 1974). Thus, to determine whether the activities found in gill and foot of *S. reidi* were substantially higher than activities present in tissues of animals from sulfide-free habitats, we compared rates of sulfide oxidation of assay mixtures containing tissue extracts from *S. reidi* with those found for extracts from tissues of other species and with solutions of known types of proteins (Table II). Tissues from *S. reidi* had the highest levels of activity, especially the gill. The foot tissue of *S. reidi* had a capacity for oxidizing sulfide that was significantly higher (t-test, $P = 0.05$) than that of the other bivalve studied, *Mercenaria mercenaria*. Homogenates of the

TABLE I

Sulfide oxidation activity in Solemya reidi *gills*

Sample	Spectrophotometric assay (Units × gFW^{-1})	Sulfide electrode assay (Units × gFW^{-1})
1	2.3	3
2	6.9	7.6
3	8.1	10

Sulfide oxidation activity was measured in *Solemya reidi* gill homogenates using two different assay techniques (details in methods). Activity is expressed in international units (μmoles of substrate converted to product per min) per gram fresh weight of tissue at 20°C.

TABLE II

Sulfide oxidizing activity

Sample	Units × gFW^{-1}	Units × ng $protein^{-1}$
S. reidi		
gill	6.38 ± 3.47 (9)	64.4 ± 35.0 (9)
foot	0.38 ± 0.07 (6)	5.3 ± 0.7 (6)
foot surface	0.88 (1)	12 (1)
M. mercenaria		
gill	0.33 ± 0.06 (2)	4.7 ± 0.2 (2)
foot	0.22 ± 0.04 (2)	2.7 ± 0.1 (2)
foot surface	0.23 (1)	4.4 (1)
adductor	0.23 ± 0.03 (2)	4.3 ± 1.4 (2)
mantle	0.20 ± 0.05 (2)	2.9 ± 1.3 (2)
S. altivelis		
muscle	0.26 (1)	1.7 (1)
Protein solutions		
albumin (bovine serum)		1.4
trypsin		1.3
myoglobin		1.2
chymotrypsin		0.9
RNAse A		0.9
poly-l-lysine		0.3

Sulfide oxidation activity of tissue supernatants and protein solutions measured using the spectrophotometric assay. Values expressed as mean ± standard deviation (number of individuals). Protein concentration in assay mixtures of protein solutions was 1 mg/ml (n = 1 for protein solutions).

entire foot failed to give a true picture of the foot's sulfide oxidation potential, however. Cytochemical staining (see below) revealed that the surface layer of the foot of *S. reidi* was where most of the sulfide oxidizing activity was found, so the real ability of the foot to oxidize (detoxify) sulfide is better indicated by the activity obtained using homogenates of tissue taken from the surface of the foot. Cytochemical staining of the other bivalves studied failed to reveal surface layers of activity. For foot of *S. reidi,* the surface layer contained sulfide oxidizing activity up to twice those of whole foot homogenates. For foot of *M. mercenaria,* the single experiment comparing whole foot *versus* foot surface activities showed no difference in rates. White skeletal muscle tissue of the marine teleost fish, *Sebastolobus altivelis,* had roughly the same sulfide oxidizing capacity as the tissues of *M. mercenaria.*

With our benzyl viologen assay system, approximately 0.25 units of sulfide oxidizing activity per g fresh weight of tissue appear to reflect a common, basal level of sulfide oxidizing activity in many animal tissues. This basal level of activity could reflect relatively non-specific catalysis of sulfide oxidation by proteins and metal ions. All of the purified proteins studied gave measurable rates of sulfide oxidizing activity (Table II). These rates were the lowest rates of sulfide oxidation found, but, assuming a tissue protein content of approximately 150 mg protein per g fresh weight of tissue, the predicted rate of sulfide oxidation due to "protein" would be about 0.2 units per g fresh weight of tissue.

Evidence for an enzymatic basis of sulfide oxidation in S. reidi

The sulfide oxidizing activity in extracts of *S. reidi* foot and gill tissue exhibited Michaelis-Menten kinetics (Fig. 1). The K_m of gill extract was 1.01 ± 0.08 (S.E.). The

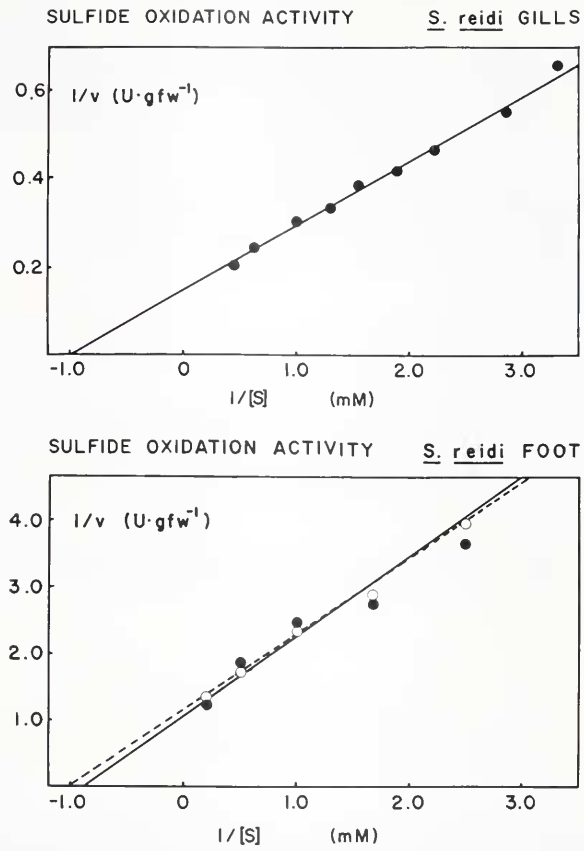

FIGURE 1. Double-reciprocal (Lineweaver-Burk) plots of sulfide oxidation activity of *Solemya reidi* gill (Top) and foot (Bottom) supernatants. Activities are expressed as international units per g fresh weight ($U \cdot gFW^{-1}$). For Figure 1B (Bottom), open circles and dashed line (– – –) represent 8.3 mg fresh weight used per assay, and closed circles and solid line (——) represent 4.2 mg fresh weight used per assay. Best fit straight lines were calculated using a weighted linear regression program.

K_m of an extract prepared from the surface of foot tissue was 1.00 ± 0.17 when 8.3 mg fresh weight were used and 1.20 ± 0.41 when 4.2 mg fresh weight were used. Protease treatment of gill supernatant from *S. reidi* resulted in up to an 80% loss in sulfide oxidizing activity, and boiling for 30 min caused a 50% loss of activity (Table III). Electrophoresis of *S. reidi* gill homogenates on native acrylamide gels, followed by sulfide oxidation activity staining, resulted in a single band in the separating gel exhibiting activity, as well as a band at the interface between the stacking and separating gel (data not shown). These results suggest that the sulfide oxidizing activity in *S. reidi* is due to the activity of one or more "sulfide oxidase" enzyme systems.

In contrast to the results obtained with extracts of tissues from *S. reidi*, the sulfide oxidizing activities of the protein solutions and the tissue extracts of animals from low-sulfide habitats showed no evidence of substrate (sulfide) saturation at sulfide concentrations up to 10 m*M* (the highest concentration compatible with the benzyl viologen assay system). These sulfide oxidizing activities appear not to be due to a specific "sulfide oxidase" enzyme having Michaelis-Menten kinetics.

The factor responsible for the activity is probably either membrane bound or enclosed within membranes; inclusion of 0.1% Triton X-100 in the homogenization

TABLE III

Susceptibility of sulfide oxidizing activity to protease treatment and boiling

Sample	Units × gFW^{-1}
Untreated *S. reidi* gill	3.3
S. reidi gill, no protease (21 h, 37°C)	1.4
S. reidi gill + chymotrypsin (21 h, 37°C)	0.65
S. reidi gill + trypsin (21 h, 37°C)	0.53
S. reidi gill + pronase (21 h, 37°C)	0.60
S. reidi gill, boiled (30 min)	1.7

Sulfide oxidation activity was measured using the benzyl viologen assay. Decrease in activity at 37°C without added protease may be due to endogenous proteases.

buffers resulted in increased levels of sulfide oxidizing activity in 10,000 $g \times 10'$ supernatants, and inclusion of 0.1% Triton X-100 in electrophoresis buffers and gels caused more of the activity to enter the separating gel.

Cytochemical localization of sulfide-oxidation activity

Since the gill homogenates contained the highest levels of sulfide oxidation activity, and the gills contained vast numbers of endosymbiotic bacteria, we wished to test the

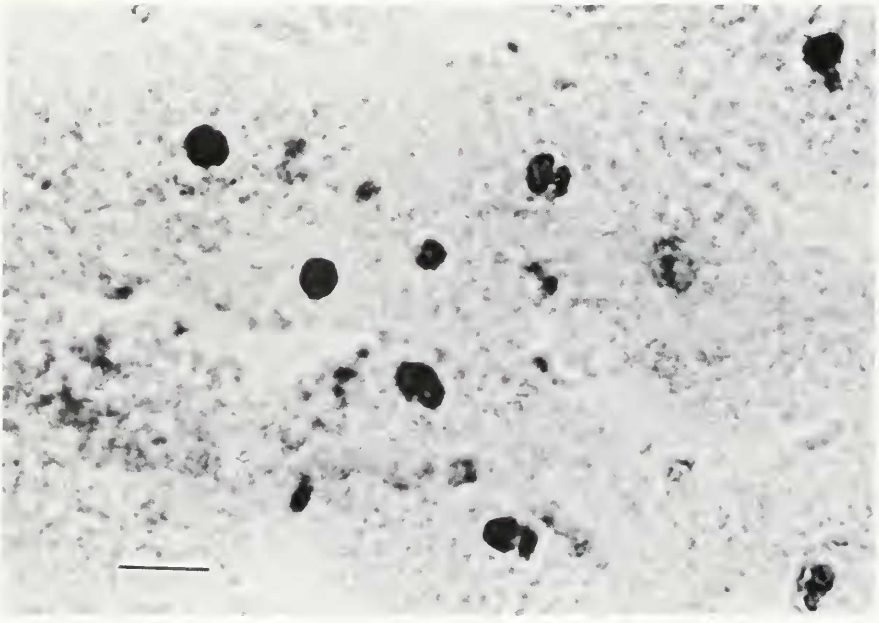

FIGURE 2. Sulfide oxidation activity staining of 6 μm section of *Solemya reidi* gill. Scale bar = 10 μm. The dark structures about 2–5 μm in diameter are the positively stained sulfide oxidizing bodies.

hypothesis that the bacteria are the site of sulfide oxidation. We modified the benzyl viologen sulfide oxidation assay to work as a cytochemical stain (see Materials and Methods). Unfixed tissue freshly cut in a cryostat was incubated with sulfide and benzyl viologen anaerobically (*i.e.*, under a coverslip). Cytochemical staining with sulfide and benzyl viologen resulted in the formation of purple color at the sites of sulfide oxidation (Fig. 2). No color was formed when benzyl viologen was added without sulfide. Staining of fixed tissue failed to show any sites of sulfide oxidation, and staining of sections cut from gill and foot tissue from two other marine bivalves, *Mytilus edulis* and *Chione* sp., also failed to show sulfide oxidizing activity (data not shown).

The bacteria in the gills of *S. reidi* did not stain positively for sulfide oxidizing activity. Rather, staining was observed exclusively in the round organelles we term sulfide oxidizing bodies, which ranged in diameter from about 1 μm to 10 μm. The sulfide oxidizing bodies appeared yellow-brown in unstained sections. To ensure that the lack of positive staining for sulfide oxidizing activity in the bacteria was not due to a failure of the sulfide or staining reagents to penetrate the bacteria, we treated some gill sections with toluene (Harwood and Peterkofsky, 1975) and subjected others to freezing and thawing so as to increase the likelihood that the bacteria were freely permeable (see Materials and Methods). Neither treatment led to a positive sulfide oxidase stain in the bacteria, but the sulfide oxidizing bodies stained positively after both treatments.

Isolation of sulfide oxidizing bodies

Table IV shows the result of density gradient centrifugation of *S. reidi* gill homogenates, and the sulfide-oxidation activity of the resulting fractions. Sulfide-oxidation activity is found only in those fractions observed to contain sulfide-oxidizing bodies, and the sulfide-oxidation activity peaks were found in the same fractions as the greatest numbers of sulfide-oxidizing bodies. Fraction 2 contained the most highly purified sulfide-oxidizing bodies; it was about twenty-five-fold enriched in these bodies and eighty-fold enriched in sulfide oxidation activity, and about forty-fold depleted in bacteria (relative to the initial tissue homogenate prior to centrifugation). Sulfide ox-

TABLE IV

Sulfide oxidizing body isolation

Fraction	Sulfide oxidizing bodies (#/ml, $\times 10^6$)	Bacteria (#/ml, $\times 10^6$)	Sulfide oxidizing activity (units $\times \mu l^{-1}$)
1	2.8	0.7	88
2	330	0.1	3480
6	21	3.4	51
10	13	4.6	37
14	9.6	27	69
16	140	140	560
17	2.2	6.0	102
Initial homogenate	13	3.9	45

Results of representative Percoll density gradient isolation of sulfide oxidizing bodies. Fraction 1 was the bottom of the gradient. Most of the sulfide oxidizing bodies formed a dense band (fraction 2) well separated from other particulate constituents of the sample (fraction 16). Fractions 2 and 16 contained the only visible bands of particles in the gradient. Fractions not shown contained low sulfide oxidizing activity.

idizing bodies were found to be very dense particles, a fact which contributed to their simple isolation. The procedure used essentially sediments most of the sulfide oxidizing bodies away from all other particles.

^{32}S-sulfide autoradiography

^{35}S-sulfide autoradiography was performed to localize sites of sulfide metabolism. Figure 3 shows the results of exposure to ^{35}S-sulfide for 1 min.

The major concentration of ^{35}S was in the region of the gill where the clam cells contained sulfide oxidizing bodies but lacked bacteria (Region 2, Fig. 4). Longer incubations in ^{35}S-sulfide (isolated gills for 10 min and intact animals for 1 h), resulted in more diffuse labeling patterns with no obvious concentration of ^{35}S. This finding could be caused either by slow oxidation of sulfide occurring throughout the gills or by the translocation of ^{35}S-labeled sulfur compounds from the initial sites of oxidation to other regions of the gill cells.

Figures 4 and 5, light and electron micrographs of sections through gill tissue, respectively, show the sulfide oxidizing bodies, the bacteria, and the locations in the gill where the bacteria and sulfide oxidizing bodies were found. Sulfide oxidizing bodies are found throughout the gill. They are most concentrated in Region 2 in Figure 4. Sulfide oxidizing bodies were identified by their size and location within the gill.

Sulfur in S. reidi *gill tissue*

A further demonstration of the distribution of sulfur compounds in the gills of *S. reidi* was provided by X-ray microanalysis of thin sections. This method detects all chemical forms of sulfur present. The X-ray microanalyses revealed high concentrations of sulfur in the sulfide oxidizing bodies, concentrations that were much higher than those in the surrounding tissue (data not shown). This finding provides further support for the role of sulfide oxidizing bodies in sulfide metabolism. Sulfur was not found in or near the bacteria at levels higher than the background levels in the surrounding cytoplasm lacking sulfide-oxidizing bodies. However, TEM examination of gill sections revealed structures in the bacteria similar in appearance to the sulfur globules found within the bacteria of another symbiont-containing clam *Lucina floridana* (Vetter, 1985).

Sulfide oxidation activity in S. reidi *foot tissue*

Figure 6 shows the results of staining transverse sections of *S. reidi* foot tissue for sulfide oxidizing activity. Only the cells comprising the surface layer of the foot stained positively with the benzyl viologen stain. No staining occurred when either sulfide or BV were applied alone. Unlike the staining observed in the gill tissue, the staining in the foot tissue is not restricted to particular observable structures; activity seems to be distributed throughout the cells. Examination of foot tissue of *S. reidi* by transmission electron microscopy failed to reveal bacteria.

Discussion

A major objective of this study was to determine where the initial steps of sulfide oxidation occur in this symbiosis. The data we have presented suggest strongly that in gill tissue of *S. reidi*, the first steps in sulfide oxidation occur not in the bacterial symbionts, but in intracellular organelles we have termed sulfide oxidizing bodies. These organelles were the only sites within the gills to stain positively for sulfide-oxidizing activity, and they were the major site of sulfur compound accumulation as

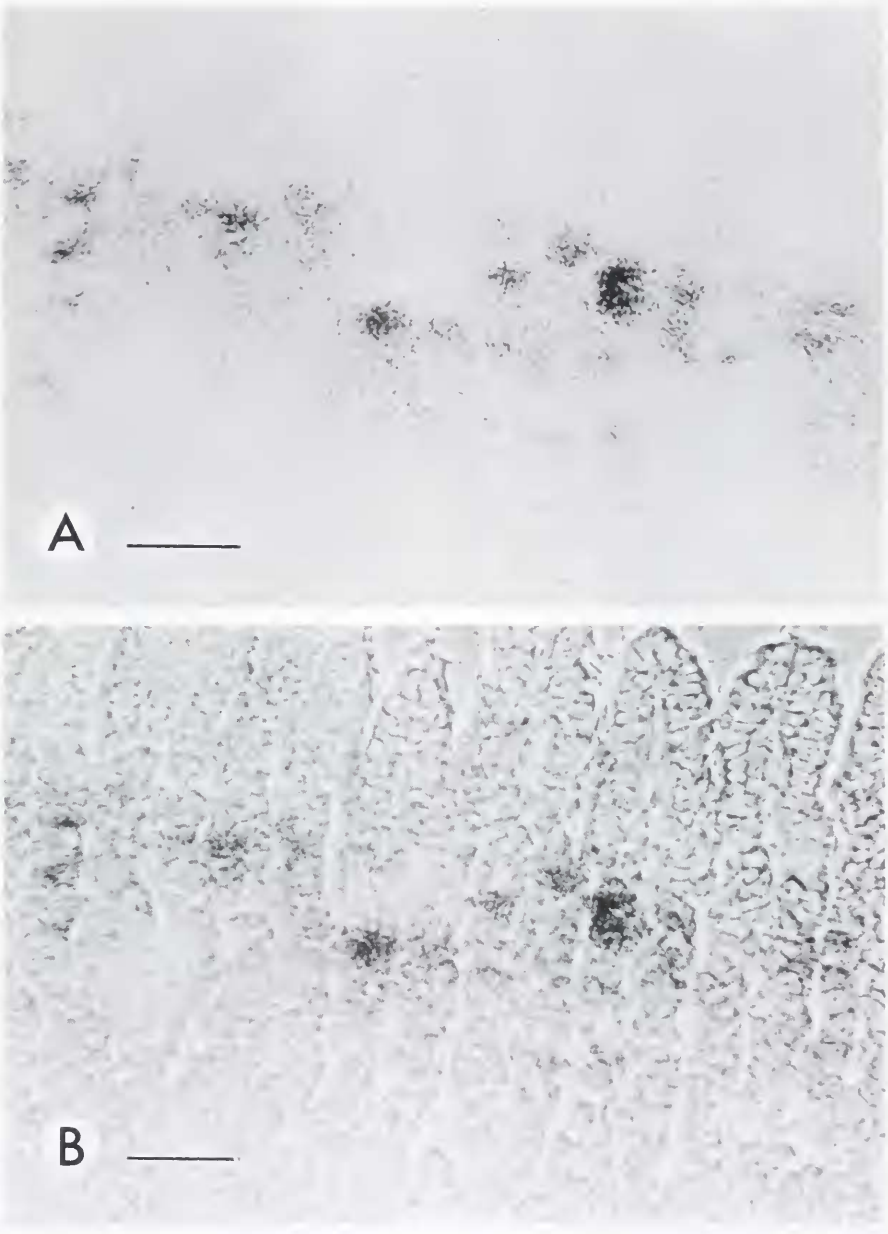

FIGURE 3. Results of a 1 min incubation of *Solemya reidi* gill in ^{35}S-sulfide. The tissue was then sectioned, exposed, and developed as described in methods. Scale bar = 50 μm. (A) Bright-field image of unstained section (same field of view as Fig. 3B). Tissue topography is barely visible, dark spots are silver grains representing sites of label accumulation. (B) Phase-contrast image of unstained section (same field of view as Fig. 3A). Tissue topography is visible, dark spots are silver grains.

observed with autoradiography and X-ray microanalysis. In addition, on density gradients the sulfide oxidizing bodies and sulfide oxidizing ability copurified; the bacterial fraction in the gradient was low in sulfide oxidizing activity.

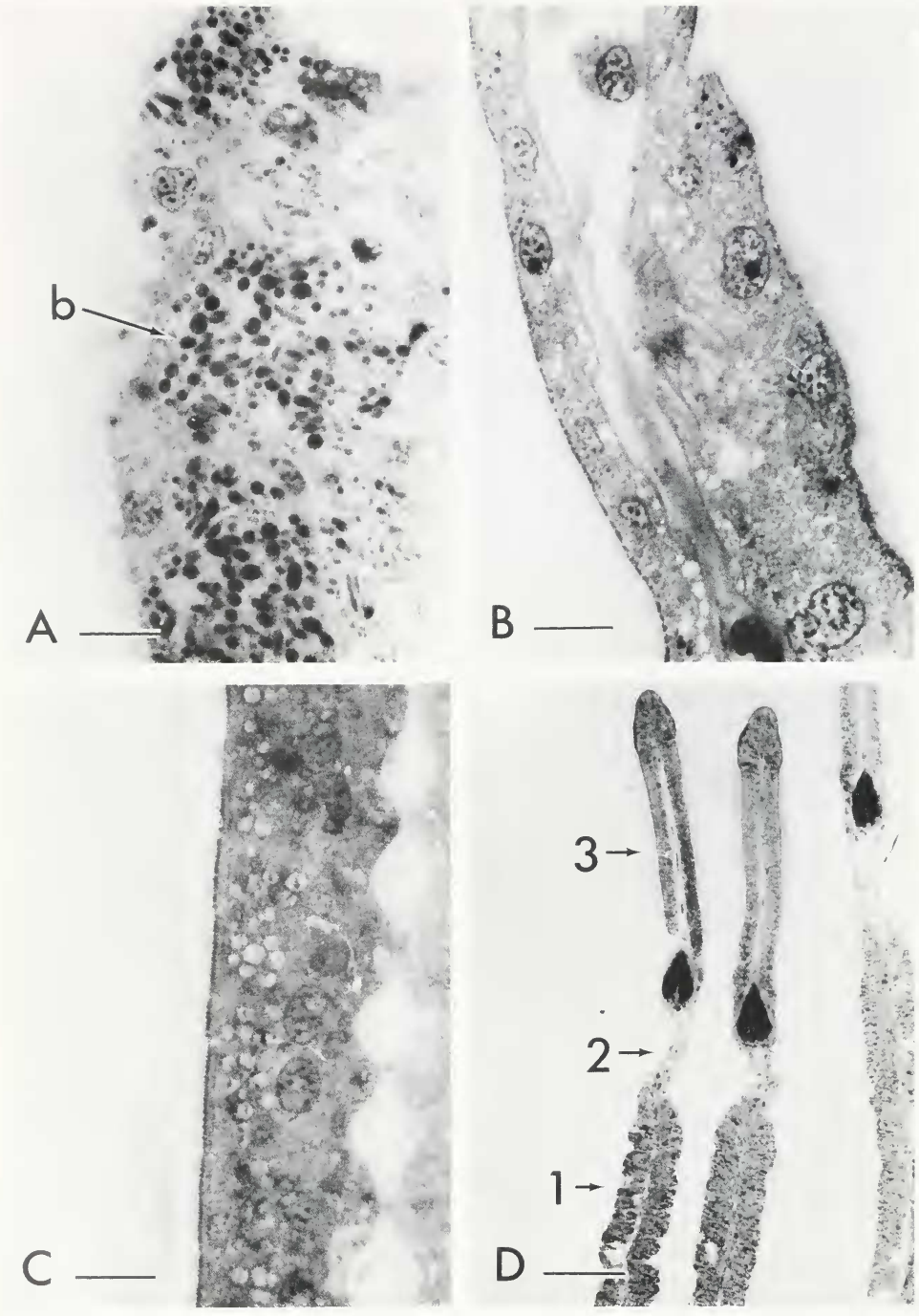

FIGURE 4. Light micrographs of 1 μm transverse section through *Solemya reidi* gill. The section was stained with methylene blue. A—Detail of bacteria-containing portion of gill, b indicates darkly stained bacteria, scale bar = 10 μm. B and C—Detail of portions of the gill which lack bacteria, scale bar = 10 μm. D—Low magnification view of three lamellae of the gill, 1 indicates the position of the types of cells shown in A, 2 indicates the position of the types of cells shown in B, and 3 indicates the position of the types of cells shown in C; the obvious dark object between 2 and 3 is the chitinous skeletal element found at the border between the filaments (3) and the subfilamental region (1 and 2); scale bar = 100 μm.

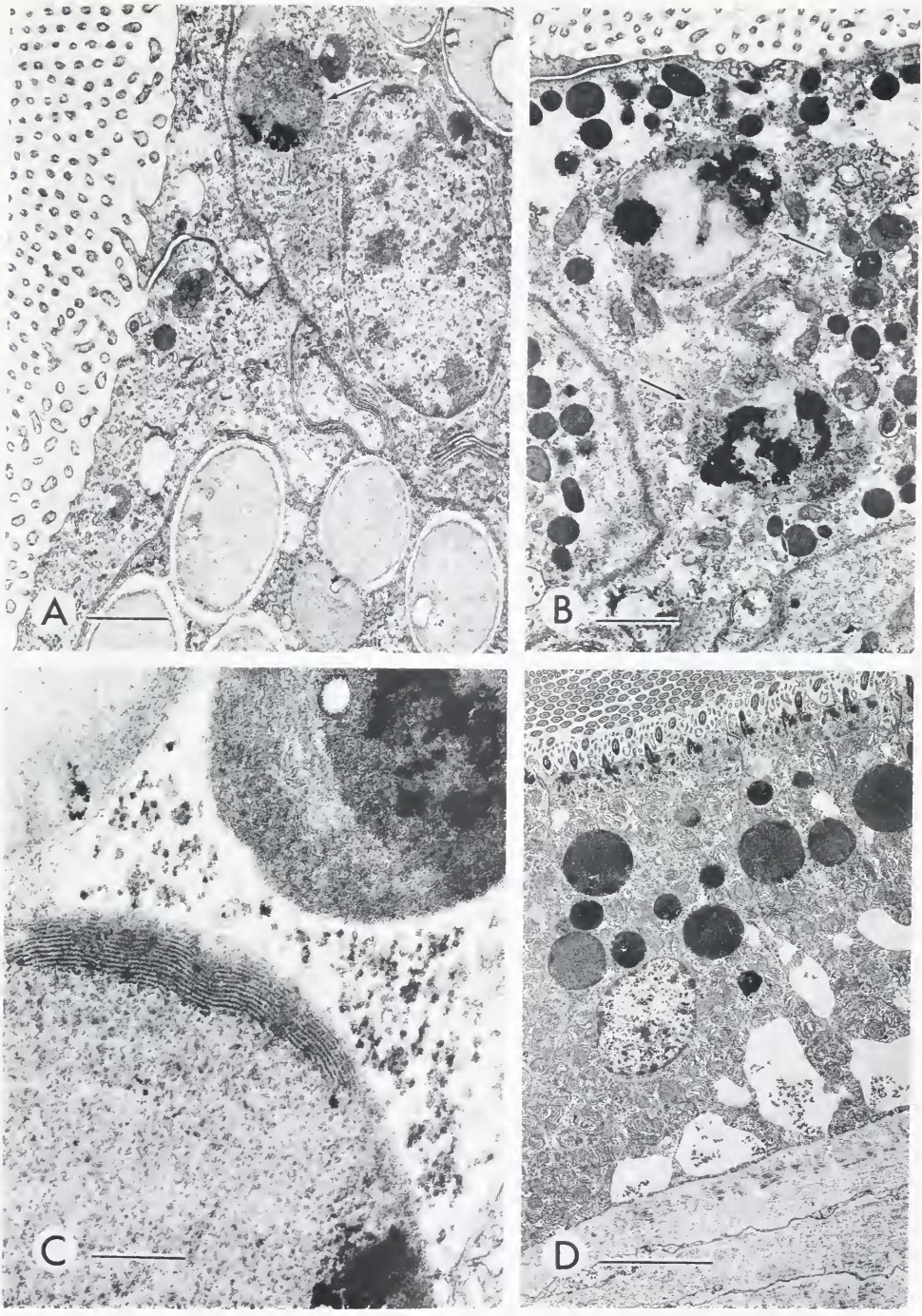

FIGURE 5. Electron micrographs of transverse sections through *Solemya reidi* gill. A—Bacteria-containing portion of gill (1 in Fig. 4D), bacteria are obvious at the bottom and top right, arrow indicates sulfide oxidizing body, the surface of the gill is on the left; scale bar = 1 μm. Sulfide oxidizing bodies are sometimes found in the same cells as bacteria. B—Bacteria-lacking portion of gill (2 in Fig. 4D), arrows indicate sulfide oxidizing bodies, surface of the gill is at the top; scale bar = 1 μm. C—Detail of bacteria-lacking filament of the gill (3 in Fig. 4D), part of three sulfide oxidizing bodies are shown; scale bar = 0.2 μm. D—Lower magnification view of bacteria-lacking filament of the gill, sulfide oxidizing bodies are the obvious darkly stained objects, many mitochondria are also present, the ciliated surface of the filament is at the top; scale bar = 10 μm.

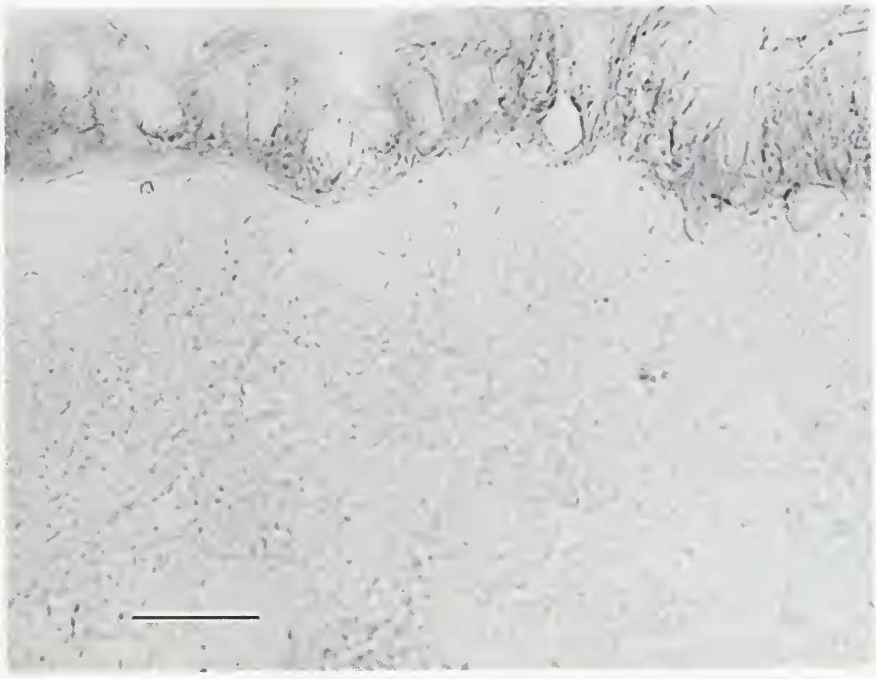

FIGURE 6. Representative sulfide oxidation activity stain of 6 μm transverse section through *Solemya reidi* foot. Scale bar = 10 μm. The dark region is positively stained epithelial cells comprising the surface of the foot. Light regions in the surface cells are unstained nuclei.

The finding that the sulfide oxidizing bodies are the apparent site of the initial oxidation of sulfide has several implications for the overall scheme of sulfur metabolism in this symbiosis. First, the rapid oxidation of sulfide to other sulfur compounds in the sulfide oxidizing bodies may serve as a means for protecting aerobic respiration of the cells from sulfide. We hypothesize that the sulfide oxidizing bodies convert sulfide that diffuses into the gill cells to less toxic or non-toxic forms of sulfur. These partially oxidized forms of sulfur are then provided to the bacteria, where the energy-yielding reactions of chemoautolithotrophic metabolism that form ATP and reducing power are thought to occur (Felbeck, 1983).

A second implication of our observations, however, is that at least some of the energy released in the oxidation of sulfide could be used directly by the animal portion of the symbiosis. We do not know what reactions occur in the sulfide oxidizing bodies, but the initial conversion of sulfide to elemental sulfur, for example, is marked by a negative free energy change of 210 kjoules/mol (Jannasch, 1984). This large negative free energy change could, in theory, provide the driving force for ATP synthesis or pyridine nucleotide reduction in the sulfide oxidizing bodies. Although the animal portion of the symbiosis appears to lack the enzymes for net CO_2 fixation (the Calvin-Benson cycle), the animals may have a substantial capacity for using energy obtained from sulfide oxidation, much as some sulfide oxidizing bacteria are capable of doing (Jannasch, 1984; Somero, 1984).

In foot tissue, where no bacterial symbionts could be detected, all of the sulfide oxidizing activity detected with the cytochemical benzyl viologen method was localized in the superficial cell layer. We hypothesize that in foot tissue, and in other tissues

lacking symbionts, a "peripheral defense" strategy for prevention of sulfide inhibition of aerobic respiration may be used. All sulfide diffusing into the tissue is rapidly and quantitatively oxidized to a non-toxic sulfur compound. The foot tissue presumably requires protection because it contains high levels of cytochrome c oxidase (Hand and Somero, 1983), which was greater than 90% inhibited by 5 μM sulfide (data not shown). It remains to be determined whether the energy released in these oxidations can be used to drive ATP synthesis or to reduce pyridine nucleotides; moreover, the fate of the oxidized sulfur compounds remains to be determined. Powell *et al.* (1979, 1980) described a similar case of body-wall sulfide oxidation as a mechanism of sulfide resistance in marine invertebrates lacking symbionts and inhabiting sulfide-rich zones.

The systems responsible for the initial steps in sulfide oxidation in foot and gill of *S. reidi* may be specific "sulfide oxidase" enzymes. This conjecture is based on the observation that extracts of these two tissues contained sulfide oxidizing systems that displayed Michaelis-Menten kinetics. Other tissues tested, as well as the purified proteins (not sulfide oxidases) we examined, did not display this type of kinetics. The sensitivity of the sulfide oxidizing activity to protease treatment and boiling is a further argument for a protein-based activity. All tissues studied, however, showed some capacity for reducing benzyl viologen when sulfide was present. This finding is not surprising in view of previous observations that sulfide oxidation is accelerated in the presence of proteins and metal ions (Baxter and Van Reen, 1958; Sorbo, 1960; Chen and Morris, 1972; Almgren and Hagstrom, 1974). We propose, however, that in animal-bacterial symbioses from sulfide-rich environments, the flow of sulfide into the cells is so great that specific enzymes, *i.e.*, sulfide oxidases, are needed to allow the organism to cope adequately with the threat posed by sulfide and to exploit effectively the energy in sulfide. Sulfide oxidases have been found in sulfide oxidizing bacteria (Moriarty and Nicholas, 1969), but not purified and characterized. Sulfide oxidation in animal tissues has not been shown to be due to the action of specific "sulfide oxidase" enzymes. Thus, little is known about this class of enzymes.

The present study was an initial examination of where and how sulfide is metabolized in *S. reidi*, and many additional questions must be answered before a comprehensive understanding of this symbiosis is possible. It is necessary to determine if the energy released during sulfide oxidation in the sulfide oxidizing bodies is trapped in a useful form, *e.g.*, as ATP. If the animal compartment of the symbiosis is able to obtain a large amount of energy from sulfide oxidation, then the role of the sulfide oxidizing bacteria in the animal's nutrition may be less than would be true if all of the sulfide oxidation was occurring within the bacteria. The nature of the sulfur compounds "fed" to the bacteria by the animal remains to be determined as well. For the symbiont containing clam *Lucina floridana*, Vetter (1985) demonstrated that large amounts of elemental sulfur are present within the bacteria in the gills of freshly collected specimens, and that the elemental sulfur levels decrease during lengthy holding of the clams in sulfide-free seawater. He has proposed that elemental sulfur may serve as a form of energy storage which can be exploited when ambient sulfide levels are low. The same strategy may occur in *S. reidi*, where we observed sulfur globules in the bacteria, and a characteristic change in gill color during storage that resembled that found for *Lucina floridana* (authors' unpub. obs.). Elemental sulfur may be the form of sulfur "fed" to the bacteria by the host; it is the most reduced inorganic sulfur compound which is nontoxic to the host.

The existence of sulfide oxidation activity in the tissues (with and without symbionts) of sulfide-habitat animals suggests that such activity may be vital to the animals. In the symbiont-containing tissues, insufficient detoxification of sulfide might result if sulfide diffused through the animal cytoplasm to the bacteria and was oxidized only

by the bacteria. The high levels of sulfide oxidation activity found in the symbiont-containing gills relative to the symbiont-free foot may reflect high demands for reduced sulfur compounds for bacterial metabolism and the requirement that animal cells be protected from sulfide.

In conclusion, the protection of the cytochrome c oxidase systems in mitochondria of gill and foot cells of *S. reidi* appears to be affected by sulfide oxidase enzyme systems which can rapidly oxidize sulfide entering the cells into non-toxic forms of sulfur. These sulfide oxidase systems may as well serve important roles in the generation of ATP and reducing power in the animal cells, a conjecture we now are testing.

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